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S1. GENERAL

Protein concentrations were determined by Bradford assay[1] using bovine serum albumin (BSA) as the standard. The relative molecular mass and purity of enzyme samples was determined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The general methods and protocols for recombinant DNA manipulations were as described by Sambrook et al.[2] DNA sequencing was performed at the Core Facilities of the Institute of Cellular and Molecular Biology, University of Texas at Austin. All reactions involving AprD4 were performed in a Coy Anaerobic Chamber (glovebox) under an atmosphere of approximately 97% N_2 and 3% H_2 with less than 1 ppm O_2. All solvents were deaerated by bubbling with nitrogen gas before they were transferred into the glovebox. In the glovebox, the solvents were stirred open to the anaerobic atmosphere overnight before use. Tetrahydrofuran (THF) was distilled from sodium/benzophenone, and dichloromethane (CH_2Cl_2) was distilled from calcium hydride under a nitrogen atmosphere. Other anhydrous solvents were purchased from Acros chemical. ^{1}H and ^{13}C NMR spectra were recorded at 400, 500, 600 MHz and 100, 125, 150 MHz with a Varian Gemini spectrometer. Chemical shifts are reported as parts per million (ppm) relative to those of the deuteriochloroform ($CDCl_3$), 7.26 ppm for ^{1}H NMR and 77.16 ppm for ^{13}C NMR, respectively. Analytical thin layer chromatography (TLC) was performed on TLB silica gel plates (250 μm) pre-coated with a fluorescent indicator. Standard flash column chromatography procedures were followed using 40–63 μm silica gel. Visualization was effected with *p*-phosphoryl molybdic acid solution. The MS analyses were carried out at the Mass Spectrometry and Proteomics Facility of the Department of Chemistry, University of Texas at Austin.

S2. Experimental Procedures

2.1. *Materials and Abbreviations*

Benzyl viologen (BV), 5′-deoxyadenosine (5′-dAdo), dithiothreitol (DTT), ethylenediamine-tetra-acetic acid (EDTA), 3-[4-(2-hydroxyethyl)piperazin-1-yl]propane-1-sulfonic acid (EPPS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), isopropyl β-D-1-thiogalactopyranoside (IPTG), Luria-Bertani (LB), molecular sieve (MS), methyl viologen (MV), nicotinamide adenine dinucleotide hydrate (NADH), nicotinamide adenine dinucleotide phosphate hydrate (NADPH), phenylmethylsulfonyl fluoride (PMSF), tris(hydroxylmethyl)aminomethane hydrochloride (Tris•HCl), and all other reagents were obtained from commercial sources. *Escherichia coli* DH5α cells were bought from Bethesda Research Laboratories (Muskegon, MI). The vector pET28b and enzyme KOD DNA polymerase were purchased from Novagen (Madison, WI). DNA modifying enzymes (for restriction digestion and ligation), PCR primers, and the overexpression host *E. coli* BL21 star (DE3) were acquired from Invitrogen (Carlsbad, CA) and New England Biolabs (NEB, Beverly, MA). Luria-Bertani (LB) media are products of Difco (Detroit, MI) or Fisher Scientific (Pittsburgh, PA). Pre-stained protein markers were bought from NEB. Kits for DNA gel extraction and spin miniprep were obtained from Qiagen (Valencia, CA). All reagents for SDS-PAGE and Amicon and Microcon YM-10 filtration products were purchased from Bio-Rad (Hercules, CA) and Millipore (Billerica, MA), respectively. Paromamine (**11**) was obtained by acidic hydrolysis of paromomycin.[3] SAM and [5-$^2H_2$]-SAM were prepared as previous described.[4,5]

2.2. *Cloning and Expression of Streptomyces tenebrarius AprD3*

The apromycin/tobramycin producer *S. tenebrarius* was obtained from the Agricultural Research Service of the US Department of Agriculture as a lyophilized sample. It was inoculated into 10 mL of

tryptic soy broth (TSB) starter culture,[6] and the resulting solution was incubated overnight at 30 °C with shaking at 250 rpm. The following day, 0.1 mL of the starter culture was inoculated into 10 mL of fresh TSB, and the resulting culture was incubated at 30 °C for 72 hr. Chromosomal DNA was extracted from 1.5 mL of this culture using a Qiagen DNeasy tissue kit. The *aprD3* gene was PCR amplified from the isolated *S. tenebrarius* DNA with the following primers: 5′-CATATGGAGCAACG-GTACGTGCTGGTCACCGGGGCCAGTCGCGGTCTGGG-3' (forward), 5′-AAGCTTCATCACGACGC-CGACCACCGCGGGACGGGGGGGCGG-3' (reverse), and cloned into a pCR:blunt vector (Invitrogen) prior to excision with *NdeI* and *HindIII* ligation into the pET28b(+) vector (Invitrogen). The resulting expression plasmid, pET28b(+):*aprD3*, was used to transform *E. coli* BL21 cells for expression of the native, recombinant AprD3. An overnight culture of *E. coli* transformed with the pET28b:*aprD3* plasmid was grown at 37 °C in LB medium containing 34 g/mL kanamycin, and used to inoculate 6 x 1 L of the same medium in a 100-fold dilution. Then 1 L cultures were incubated at 37 °C until cell had reached an optical density of 0.6 at 600 nm. At which point AprD3 expression was induced by 1mM IPTG. The cells were harvested by centrifugation at 20000 x *g* for 30 min, thawed and suspended in 50 mM EPPS (pH 8.0) with 20 mM imidazole and 300 mM NaCl. After sonication (8 x 1 min), the lysate was spun at 26500 x *g* for 40 min. 1 mM PMSF was added. The supernatant was mixed with 8 mL of Ni-NTA resin at 4 °C. The slurry was transferred to an empty column and washed with 100 mL of 50 mM EPPS buffer (pH 8.0) containing 300 mM NaCl and 20 mM imidazole. The protein was eluted with 50 mL of 50 mM EPPS buffer (pH 8.0) containing 300 mM NaCl and 300 mM imidazole. The purified protein was dialyzed twice against 1 L of 50 mM EPPS buffer (pH 8.0) containing 300 mL NaCl. The dialyzed protein was frozen with liquid nitrogen and stored at –80 °C until use.

2.3. *Cloning, Expression and reconstitution of Streptomyces tenebrarius AprD4*

The aprD4 gene was PCR amplified from the isolated *S. tenebrarius* DNA with the following primers: 5'-CATATGCGACGAATGCGGCTCGGCACGGTAC-3' (forward), 5'-AAGCTTCATCAGGCG-TCACCGGTCGACCAGGCGTG-3' (reverse), and cloned into a pCR:blunt vector (Invitrogen) prior to excision with *NdeI* and *HindIII* and ligation into the pET28 vector (Invitrogen). The resulting expression plasmid, pET28:*aprD4*, was used to transform *E. coli* Rosetta DE3 (Novagen) for expression of native, recombinant AprD4. Transformants were grown in 6 x 1 L of LB medium with 34 g/mL kanamycin and 68 g/mL chloramphenicol at 37 °C with shaking at 200 rpm. AprD4 expression was induced with 1 mM IPTG when the cells had reached an optical density of 0.6 at 600 nm. Cells were harvested by centrifugation at 4500 x *g* for 20 min, thawed and suspended in 50 mM Tris buffer (pH 8.0, HCl) with 20 mM imidazole. After sonication (8 x 1 min), the lysate was spun at 26500 x *g* for 30 min. Lysate was loaded on a Ni-NTA column (8 mL) pre-equilibrated with 50 mM Tris buffer (pH 8.0, HCl) and 20 mM imidazole. After washing with 10 mL of 50 mM Tris buffer (pH 8.0, HCl) and 20 mM imidazole, the column was developed with 8 mL of 50 mM Tris buffer (pH 8.0, HCl) and 40 mM imidazole, 8 mL of 50 mM Tris buffer (pH 8.0, HCl) and 100 mM imidazole, and 8 mL of 50 mM Tris buffer (pH 8.0, HCl) and 300 mM imidazole. The fractions were collected and centrifuged with a YM-10 filter at 4500 x *g* for 1 min. The protein was dialyzed twice against 1 L of 50 mM Tris buffer (pH 8.0, HCl). The dialyzed protein was frozen with liquid nitrogen and stored at –80 °C until reconstitution with iron and sulfur.

Reconstitution followed the previously published procedure.[7] Approximately 3–5 mg of AprD4 was thawed, and transferred into a conical vial. The vial was transferred to the glovebox. The enzyme was diluted with 20 mM NH_4HCO_3 buffer (pH 7.8) containing 5 mM DTT to a volume of ca. 2 mL and gently stirred at ca. 10 °C in an open vial for ca. 2 hr in order to equilibrate with the anaerobic atmosphere. The solution was made 1.88 mM in dithiothreitol and allowed to stir for 15 min. To this solution was

slowly added 42 μL of a 50 mM solution of $Fe(NH_4)_2(SO_4)_2$ in anaerobic water over 10 min. After 10 min, 42 μL of a 50 mM solution of Na_2S in anaerobic buffer containing 20 mM NH_4HCO_3 (pH 7.8) and 5 mM DTT was added over 10 min. The solution was gently stirred at ca. 10 °C for 2 hr. The reconstituted enzyme was loaded onto a 50 mL Sephadex G-25 column pre-equilibrated with anaerobic buffer containing 20 mM NH_4CO_3 (pH 7.8) and 5 mM DTT, and the column was eluted with the same buffer. Protein fractions were collected, concentrated to approximately 100 μM of enzyme and stored in the glovebox at ca. 10 °C until use. Iron content in the reconstituted AprD4 was assessed using the ferrozine assay,[8] and the sulfide content was determined by the method devised by Helmut Bienert.[9] The iron and sulfide content reported in the text is an average of three measurements.

2.4. *AprD4 Activity Assays and AprD3 Activity Assays*

The reaction mixture contained the following: 50 mM NH_4HCO_3 (pH 7.8), 10 mM DTT, 1 mM paromamine (**11**), 2 mM enzymatically prepared SAM, 2 mM sodium dithionite, and 0.01 mM AprD4. Assays were initiated by the addition of AprD4, incubated for 8 hr at room temperature, and quenched by passage through YM-10 Microcon centrifugal filters to remove enzymes. The filtrate of each sample was frozen and stored at –80 °C until analysis of HPLC and mass spectroscopy.

To find proper reducing systems for the AprD4 reaction, all reactions were treated with 1 mM of the chemical reductants being tested. When flavodoxin and flavodoxin reductase from *E. coli* (obtained as previously described[10]) were used as the reducing system, the protein concentration was 0.1 mM.

To an assay mixture of AprD4, 0.01 mM AprD3 and 1 mM NAD(P)H were added. Assays were incubated for 1 hr at room temperature, quenched by passage through YM-10 Microcon centrifugal filters to remove enzymes. The filtrate of each sample was frozen and stored at –80 °C until analysis of HPLC and mass spectroscopy.

2.5. *HPLC Detection of 5'-Deoxyadenosine (5'-dAdo)*

A 20 μL aliquot of the quenched AprD3/AprD4 reaction or AprD4 reaction described above was analyzed using HPLC equipped with a Varian Microsorb-MV 100-5 C18 (4.6 x 250 nm) column pre-equilibrated in H_2O containing 0.1% trifluoroacetic acid (solvent A). After sample loading, the column was eluted with a linear gradient from 0–20% acetonitrile containing 0.1% trifluoroacetic acid (Solvent B) over 30 min. The UV detector was set at 260 nm. An authentic, commercially obtained 5'-dAdo was injected as a retention time standard for each. The stoichiometric ratio reported in the text is an average of three measurements.

2.6. *HPLC Detection of AprD4/AprD3 Reaction Product*

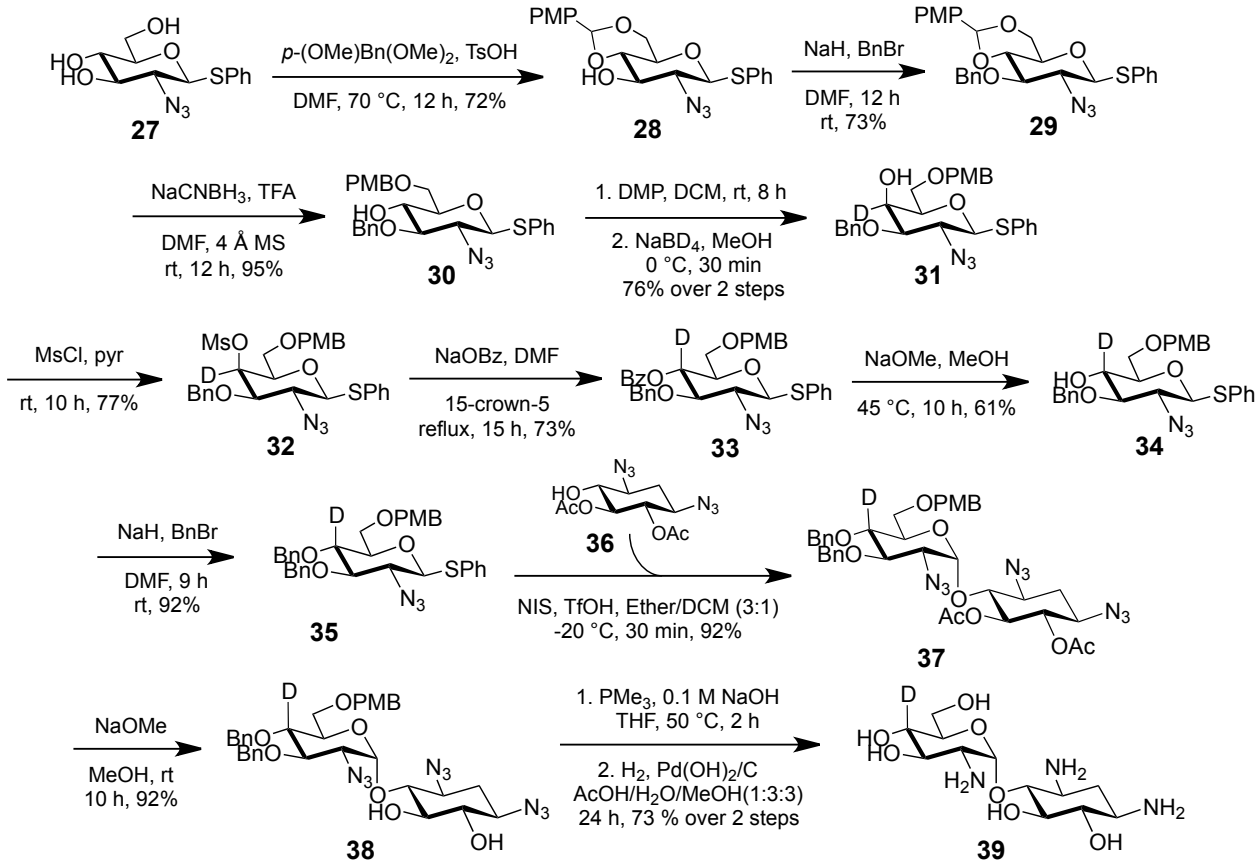
A 10 μL aliquot of the quenched AprD4/AprD3 reaction or the AprD4 reaction was analyzed using HPLC equipped with a Varian Microsorb-MV 100-5 C18 (4.6 x 250 nm) and eluted with H_2O containing 0.4% trifluoroacetic acid for 15 min. The elution was monitored by Corona® charged aerosol detector. An authentic paromamine was injected as a retention time standard.

2.7. *Mass Spectroscopic Analysis of AprD3/AprD4 Activity and AprD4 Activity*

Assay mixtures were diluted in water and injected directly into an Agilent 6350 Accurate Mass QTOF-MS at 0.15 mL/min. The analyte was ionized by electrospray ionization and detected in positive ion mode.

2.8. *Synthesis of [4′-^{2}H]-paromamine (**39**)*

[4′-^{2}H]-Paromamine (**39**) was prepared from **27** as summarized below. Intermediate **30** was obtained from **27**[11] via 4,6-diol protection with anisaldehyde dimethyl acetal (**27**→**28**), followed by benzyl protection of the 3-OH group (**28**→**29**) and selective reduction using sodium cyanoborohydride and trifluoroacetic acid.[12] Equatorial introduction of deuterium at C4 was achieved via Dess-Martin oxidation and sodium borodeuteride reduction (**30**→**31**).[13] The stereochemistry at C4 of **31** was inverted in a two-step procedure with sodium benzoate serving as a nucleophile under reflux conditions (**31**→**32**→**33**).[14] Alkaline hydrolysis of the benzoate protecting group in **33** followed by benzyl protection of the exposed hydroxyl at C4 provided **35**. Intermediate **35** was then coupled with an azide derivative of 2-deoxystreptamine (**36**)[15] in the presence of *N*-iodosuccinimide and trifluoromethanesulfonic acid to provide the pseudodisaccharide **37**. Next, the acetyl protecting groups were removed by alkaline hydrolysis to give **38**, and the azide groups were converted to amines using Staudinger reduction.[15] Final deprotection of benzyl group by hydrogenolysis provided **39** in 7.4% yield over 13 steps.



[Abbreviations: DMP, Dess-Martin periodinane; PMP, *p*-methoxyphenyl; PMB, *p*-methoxybenzyl; Ms, methanesulfonyl]

*2.8.1. Synthesis of compound **28***

Compound **27** was prepared as previously described.[11] Compound **27** (12.88 g, 43.32 mmol) and anisaldehyde dimethyl acetal (9.14 mL, 53.72 mmol) were dissolved in DMF (200 mL). *p*-Toluenesulfonic acid monohydrate (0.745 g, 4.33 mmol) was added and the flask was attached to a rotary evaporator, rotated, evacuated, and lowered into a water bath at approximately 60 °C to remove the methanol, which was formed during the reaction. After 1 hr, triethylamine was added and

all solvent was removed by evaporation. The residue was diluted with ethyl acetate (200 mL) and saturated aqueous sodium bicarbonate solution (100 mL). The aqueous layer was extracted with dichloromethane (3 x 200 mL). The combined organic extracts were washed with brine, dried over magnesium sulfate, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel (Hex/EtOAc = 2:1) to afford compound **28** (13.50 g, 72%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.59–7.56 (m, 2H, Bn), 7.39–7.26 (m, 5H, Bn), 6.90–6.88 (m, 2H, Bn), 5.49 (s, 1H, PMPCH), 4.55 (d, *J* = 10.2 Hz, 1H, H-1), 4.36 (dd, *J* =10.2, 4.5 Hz, 1H, H-4), 3.80 (s, 3H, OCH_3), 3.79–3.74 (m, 2H, H-6) 3.47–3.44 (m, 2H, H-5, H-3), 3.36 (dd, *J* = 10.1, 9.0 Hz, 1H, H-2), 2.68 (d, *J* = 2.72 Hz, 1H, OH). ^{13}C NMR (150 MHz, CD_3OH) δ 161.7, 134.9, 132.5, 130.8, 130.6, 130.0, 129.2, 115.2, 103.2, 88.0, 81.6, 75.2, 71.7, 69.7, 66.6, 56.7. HRMS (ESI, positive ion mode) calcd for $C_{20}H_{21}N_3O_5S^+$ [*M* + *Na*]$^+$ 438.1100, found 438.1093.

*2.8.2. Synthesis of compound **29***

To a solution of compound **28** (7.55 g, 18.17 mmol) in anhydrous DMF (120 mL) at 0 °C under nitrogen atmosphere, sodium hydride (60% in mineral oil, 0.945g, 23.62 mmol) was added. The mixture was warmed to room temperature and was stirred for 30 min. The mixture was cooled to 0 °C and benzyl bromide (2.81 mL, 23.62 mmol) was added dropwise. The reaction was then allowed to stand for 12 hr at room temperature. The mixture was treated with methanol at 0 °C and all the solvent was evaporated under reduced pressure. The residue was diluted with dichloromethane (200 mL) and washed with saturated aqueous sodium bicarbonate solution (100 mL). The aqueous layer was extracted with dichloromethane (3 x 100 mL). The combined organic extracts were washed with brine, dried over magnesium sulfate, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 3:1) to afford compound **29** (6.68 g, 73 %) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.58–7.56 (m, 2H, Ph), 7.42–7.40 (m, 2H, Ph), 7.39–7.30 (m, 8H, Ph), 6.96–6.91 (m, 2H, Ph), 5.54 (s, 1H, PMPCH), 4.92 (d, *J* = 10.9 Hz, 1H, $PhCH_2$), 4.79 (d, *J* = 11.0 Hz, 1H, $PhCH_2$), 4.50 (d, *J* = 10.2, 1H, H-1), 4.38 (dd, *J* = 10.5, 5.5 Hz, 1H, H-4), 3.83 (s, 3H, OCH_3), 3.78 (t, *J* = 10.3 Hz, 1H, H-3), 3.65–3.61 (m, 2H, H-6), 3.47–3.43 (m, 1H, H-5), 3.37 (dd, *J* = 10.3, 8.8 Hz, 1H, H-2).^{13}C NMR (125 MHz, $CDCl_3$) δ 160.2, 137.9, 130.7, 129.6, 129.1, 128.7, 128.5, 128.3, 128.0, 127.3, 113.7, 101.3, 86.6, 81.3, 81.0, 75.2, 70.5, 68.5, 64.7, 55.3. HRMS (ESI, positive) calcd for $C_{27}H_{27}N_3O_5S^+$ [*M* + *Na*]$^+$ 528.1569, found 528.1559.

*2.8.3. Synthesis of compound **30***

A mixture of compound **29** (4.544 g, 8.99 mmol), sodium cyanoborohydride (4.518 g, 71.91 mmol), and freshly activated 4 Å molecular sieve (1 g) in dry DMF (90 mL) was stirred at room temperature under nitrogen for 30 min. Trifluoroacetic acid (6.87 mL, 89.88 mmol) was added dropwise at 0 °C. After stirring overnight at 12 hr, the reaction mixture was filtered through Celite pad and poured into ice-cold saturated aqueous sodium bicarbonate solution (100 mL). The mixture was diluted with ethyl acetate (200 mL), washed with saturated aqueous sodium bicarbonate solution (50 mL), brine, dried over magnesium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 3:1) to give compound **30** (4.326 g, 95%) as a clear oil. Compound **30** was unstable, only mass spectra were measured. HRMS (ESI, positive) calcd for $C_{27}H_{29}N_3O_5S^+$ [*M* + *Na*]$^+$ 530.1726, found 530.1718.

*2.8.4. Synthesis of compound **31***

To a mixture of compound **30** (4.326 g, 8.52 mmol) in dry dichloromethane (100 mL) was added Dess-Martin periodinane (7.229 g, 17.04 mmol). After stirring for 12 hr under nitrogen gas at room temperature, mixtures (60 mL) of 10% aqueous sodium thiosulfate solution and saturated aqueous sodium bicarbonate solution (1:1) were added to the reaction mixture. The reaction was allowed to stir for an additional 30 min. The organic layer was washed with aqueous sodium thiosulfate/sodium bicarbonate (1:3) solution (3 x 60 mL) and then brine, dried over magnesium sulfate, filtered, and concentrated *in vacuo*. The crude ketone compound was dissolved in anhydrous methanol (50 mL) and dry dichloromethane (50 mL). To this solution was added sodium borodeuteride (0.428 g, 10.23

mmol) portionwise at 0 °C. After stirring for 10 min at 0 °C, the reaction mixture was stirred at room temperature for an additional 3 hr. The reaction was quenched by the addition of saturated aqueous ammonium chloride solution (50 mL), and the aqueous layer was extracted with dichloromethane. The combined organic layers were dried over magnesium sulfate, filtered, and concentrated under reduced pressure. Purification by flash column chromatography on silica gel (Hex/EtOAc = 3:1) gave compound **31** (3.294 g, 76%) as a white solid. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.59–7.57 (m. 2 H, Ph), 7.39–7.32 (m, 4H, Ph), 7.28–7.23 (m, 6H, PMP, Ph), 6.88–6.87 (m, 2H, Ph), 4.66 (dd, *J* = 24.9, 11.5 Hz, 2H, Bn), 4.49 (m, 2H, PMB), 4.36 (d, *J* = 10.2 Hz, 1H, H-1), 3.79 (s, 3H, OCH_3), 3.77–3.71 (m, 2H, H-6), 3.63 (dd, *J* = 10.2, 9.5 Hz, 1H, H-2), 3.52 (t, *J* = 5.7, 1H, H-5), 3.38 (d, *J* = 9.5 Hz, 1H, H-3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 159.3, 136.9, 133.0, 131.6, 129.8, 129.4, 128.9, 128.5, 128.2, 128.1, 128.0, 113.8, 86.2, 80.9, 73.3, 71.8, 68.9, 60.9, 55.2. HRMS (ESI, positive) calcd for $C_{27}H_{28}DN_3O_5S^+$ [*M* + *Na*]$^+$ 531.1788, found 531.1780.

*2.8.5. Synthesis of compound **32***

To a solution of compound **31** (2.332 g, 4.59 mmol) in anhydrous pyridine (40 mL) at 0 °C were added methanesulfonyl chloride (1.77 mL, 22.93 mmol) and dimethylaminopyridine (0.028 g, 0.23 mmol). The reaction mixture was stirred at room temperature for 10 hr. Methanol (10 mL) was added to quench the reaction at 0 °C. The mixture was concentrated under reduced pressure and co-evaporated with toluene (3 x 30 mL). The residue was diluted with dichloromethane (50 mL) and water (50 mL). The aqueous layer was extracted with dichloromethane (3 x 50 mL), dried over magnesium sulfate, filtered, and concentrated. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 3:1) to afford compound **32** (2.081g, 77%) as a light yellowish oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.60–7.57 (m, 2H, Ph), 7.42–7.30 (m, 10H, Ph), 6.91–6.89 (m, 2H, Ph), 4.81 (d, *J* = 10.6, 1H, Bn), 4.58 (dd, *J* = 10.7, 2.0 Hz, 2H, PMP), 4.43 (d, *J* = 10.8 Hz, 1H, Bn), 4.41 (d, *J* = 9.56 Hz, 1H, H-1) 3.81 (s, 3H, OCH_3), 3.75–3.69 (m, 3H, H-6, H-3), 3.54–3.44 (m, 2H, H-2, H-5), 2.92 (s, 3H, SCH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 159.4, 136.3, 133.4, 131.2, 130.95, 129.68, 129.98, 128.64, 128.58, 128.45, 128.41, 113.9, 86.3, 79.3, 73.5, 72.7, 67.4, 61.1, 55.3, 39.1. HRMS (ESI, positive) calcd for $C_{28}H_{30}DN_3O_7S_2^+$ [*M* + *Na*]$^+$ 609.1564, found 609.1559.

*2.8.6. Synthesis of compound **33***

To a solution of compound **32** (2.081 g, 3.55 mmol) in anhydrous dimethylforaminde (DMF, 100 mL) were added sodium benzoate (1.214 g, 10.64 mmol) and 15-crown-5 (50 μL). The reaction mixture was heated to reflux for 15 hr. The solvent was evaporated under reduced pressure, and the residue was diluted with dichloromethane (50 mL) and water (50 mL). The aqueous layer was extracted with dichloromethane (3 x 50 mL), dried over magnesium sulfate, filtered, and concentrated. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 3:1) to give compound **33** (1.316 g, 60 %) as a white solid. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.97–7.95 (m, 2H, Bz), 7.61–7.59 (m, 3H, Ph, Bz), 7.45–7.43 (m, 2H, Bz), 7.32–7.29 (m, 3H, Ph), 7.16–7.11 (m, 7H, PMB, Bn), 6.77–6.75 (m, 2H, PMB), 4.72 (d, *J* = 10.8 Hz, Bn), 4.58 (d, *J* = 10.8 Hz, Bn), 4.51 (d, *J* = 10.8 Hz, 1H, H-1), 4.41 (m, 2H, PMB), 3.76 (s, 3H, OCH_3), 3.72 (m, 1H, H-6), 3.66 (d, *J* = 9.3 Hz, 1H, H-3), 3.59 (m, 2H, H-5, H-6), 3.47 (dd, *J* = 10.1, 9.2 Hz, 1H, H-2). ^{13}C NMR (150 MHz, $CDCl_3$) δ 165.1, 159.1, 136.9, 133.5, 133.3, 131.0, 129.8, 129.7, 129.3, 129.3, 129.0, 128.4, 128.4, 128.3, 128.2, 127.9, 86.1, 82.3, 77.9, 75.3, 73.2, 69.1, 64.8, 55.2. HRMS (ESI, positive) calcd for $C_{34}H_{32}DN_3O_6S^+$ [M + Na]$^+$ 632.2051, found 635.2013.

*2.8.7. Synthesis of compound **34***

To a solution of compound **33** (2.399 g, 3.92 mmol) in anhydrous methanol (10 mL) and anhydrous dichloromethane (40 mL) was added sodium methoxide (4.37 M in methanol, 0.18 mL, 0.78 mmol) dropwise. The reaction mixture was stirred at 45 °C for 10 hr. After cooling down to room temperature, the reaction mixture was neutralized with Amberlite IR-120 (H^+ form) resin, filtered, and concentrated. The residue was purified by flash column chromatography (Hex/EtOAc = 3:1) on silica gel to give compound **34** (1.215 g, 61%) as a clear oil. Compound **34** was unstable so it was characterized only

by mass spectroscopy. HRMS (ESI, positive) calcd for $C_{27}H_{28}DN_3O_5S^+$ [*M* + *Na*]$^+$ 531.1788, found 531.1781.

*2.8.8. Synthesis of compound **35***

To a solution of compound **34** (2.283 g, 4.49 mmol) in anhydrous DMF (50 mL) at 0 °C under nitrogen atmosphere, sodium hydride (60% in mineral oil, 0.215g, 5.39 mmol) was added portionwise. The mixture was warmed to room temperature and stirred for 30 min. The solution was cooled to 0 °C and benzyl bromide (0.64 mL, 5.39 mmol) was added dropwise. The reaction was continued with stirring for 9 hr at room temperature. The mixture was quenched with methanol at 0 °C and the solvent was evaporated *in vacuo*. The residue was diluted with dichloromethane (100 mL) and washed with saturated aqueous sodium bicarbonate solution (50 mL). The aqueous layer was repeatedly extracted with dichloromethane (3 x 50 mL). The combined extracts were washed with brine, dried over magnesium sulfate, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc 4:1) to provide compound **35** (2.208 g, 82 %) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.70–7.68 (m, 2H, Ph), 7.41–7.33 (m, 13H, Ph), 7.28–7.27 (m, 2H, Ph), 6.96–6.94 (m, 2H, Ph), 4.95–4.90 (m, 2H, Bn), 4.86 (d, *J* = 10.9 Hz, 1H, Bn), 4.66–4.63 (m, 2H, PMB), 4.55 (d, *J* = 11.6 Hz, 1H, Bn), 4.50 (d, *J* = 10.1 Hz, 1H, H-1), 3.86 (s, 3H, OCH_3), 3.84-3.78 (m, 2H, H-6), 3.58 (d, *J* = 9.5 Hz, 1H, H-3), 3.53 (dd, *J* = 4.12, 2.1 Hz, 1H, H-5), 3.44 (t, *J* = 9.7, 1H, H-2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 159.3, 138.0, 137.7, 131.4, 130.3, 129.4, 129.0, 128.6, 128.5, 128,4, 128.2, 128.1, 127.92, 127.88, 113.9, 86.1, 85.1, 79.4, 75.9, 75.0, 73.2, 68.4, 65.2, 55.3. HRMS (ESI, positive) calcd for $C_{34}H_{34}DN_3O_5S^+$ [*M* + *Na*]$^+$ 621.2258, found 621.2250.

*2.8.9. Synthesis of compound **37***

Compound **36** was prepared as previously described.[15] Compound **35** (2.208 g, 3.69 mmol) and compound **36** (0.916 g, 3.07 mmol) were co-evaporated from dry toluene (500 mL) three times and further dried under high vacuum overnight. To a preactivated 4 Å MS (3 g) was added a solution of compound **35** and **36** in anhydrous diethyl ether (30 mL) and anhydrous dichloromethane (10 mL). The mixture was stirred for 30 min at room temperature, and cooled to –40 °C. *N*-Iodosuccinimide (1.797 g, 7.99 mmol) was added, and the reaction mixture was stirred for 20 min. Trifluoromethanesulfonic acid (136 μL, 1.53 mmol) was slowly added, and the reaction was warmed to –20 °C and kept stirring for 30 min. The reaction mixture was diluted with dichloromethane (100 mL), filtered through Celite pad, and washed with 10% aqueous sodium bisulfite solution (100 mL) and saturated aqueous sodium bicarbonate solution (100 mL). The aqueous layers were extracted with dichloromethane (3 x 50 mL). The combined organic phase was washed with brine, dried over magnesium sulfate, filtered and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (Hex/EtOAc = 3:2) to give compound **37** (2.212g, 92%) as a sticky liquid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33–7.23 (m, 12H, Ph), 7.13–7.11 (m, 2H, Ph), 6.83 (d, *J* = 8.4 Hz, 2H, Ph), 5.14 (t, *J* = 9.2 Hz, 1H, H-5), 5.13 (d, *J* = 4 Hz, 1H, H-1'), 4.92 (t, *J* = 10.0 Hz, 1H, H-6), 4.84 (dd, *J* = 25.0, 10.5 Hz, 2H, PMB), 4.75 (d, *J* = 10.8 Hz, 1H, Bn), 4.60 (d, *J* = 11.6 Hz, 1H, Bn), 4.47 (d, *J* = 11.2 Hz, 1H, Bn), 4.40 (d, *J* = 11.6 Hz, 1H, Bn), 4.13 (s, 1H, H-5'), 3.93 (d, *J* = 10.4 Hz, 1H, H-3'), 3.80 (dd, *J* = 10.6, 2.0 Hz, 1H, H-6'), 3.75 (s, 3H, -OCH_3), 3.66–3.58 (m, 3H, H-1, H-6', H-4), 3.43–3.37 (m, 1H, H-3), 3.35 (dd, *J* = 10.2, 3.6 Hz, 1H, H-2'), 2.40 (dt, *J* = 13.6, 4.4 Hz, 1H, H-2), 1.585 (q, *J* = 12.8 Hz, 1H, H-2). ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.8, 169.5, 159.3, 137.9, 137.8, 129.8, 129.6, 128.4, 128.3, 127.9, 127.8, 127.7, 127.6, 113.8, 99.3, 79.4, 78.2, 75.3, 74.8, 74.1, 73.5, 73.2, 71.6, 67.4, 63.1, 58.7, 57.7, 55.2, 20.6, 20.5. HRMS (ESI, positive) calcd for $C_{38}H_{42}DN_9O_{10}^+$ [*M* + *Na*]$^+$ 809.3093, found 809.3079.

*2.8.10. Synthesis of compound **38***

To a solution of compound **37** (2.196 g, 2.79 mmol) in methanol (30 mL) and dichloromethane (10 mL) was added sodium methoxide (4.37 M in methanol, 64 μL, 0.28 mmol) dropwise. The reaction mixture was stirred at room temperature for 10 hr. The reaction mixture was neutralized with Amberlite IR-120 (H^+ form) resin, filtered, and concentrated. The residue was purified by flash column

chromatography on silica gel (Hex/EtOAc = 3:2) to give compound **38** (1.215 g, 92%) as a clear oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.35–7.23 (m, 10H, Ph), 7.14–7.10 (m, 2H, Ph), 6.85–6.81 (m, 2H, Ph), 5.13 (d, *J* = 3.2 Hz, 1H, H-1), 4.88 (dd, *J* = 31.2, 10.8 Hz, 2H, PMB), 4.75 (d, *J* = 10.4 Hz, 1H, Bn), 4.60 (d, *J* = 12.0 Hz, 1H, Bn), 4.48 (d, *J* = 10.8 Hz, 1H, Bn), 4.40 (d, *J* = 11.6 Hz, 1H, Bn), 4.19 (s, 1H, OH), 4.07 (t, *J* = 2.0, 1H, H-5'), 3.96 (d, *J* = 10.4 Hz, 1H, H-3'), 3.78 (dd, *J* = 10.6, 2.8 Hz, 1H, H-6'), 3.75 (s, 3H, OCH_3), 3.66–3.62 (m, 2H, H-2', H-6'), 3.49–3.38 (m, 3H, H-4, H-5, H-1), 3.25 (dd, *J* = 5.6, 2.8 Hz, 2H, H-3, H-6), 2.33–2.28 (m, 1H, H-2), 1.49–1.45 (m, 1H, H-2). ^{13}C NMR (125 MHz, $CDCl_3$) δ 159.3, 137.8, 137.5, 129.74, 129.70, 128.5, 128.4, 128.1, 128.0, 127.9, 127.7, 113.8, 99.6, 84.0, 80.9, 75.9, 75.6, 75.3, 74.9, 73.2, 71.6, 67.5, 64.3, 59.6, 58.8, 55.2, 32.1. HRMS (ESI, positive) calcd for $C_{34}H_{38}DN_9O_8^+$ [*M* + *Na*]$^+$ 725.2882, found 725.2876.

*2.811. Synthesis of compound **39***

To a solution of compound **38** (0.259 g, 0.37 mmol) in THF (9 mL) and 0.1 M NaOH (3 mL) was added trimethylphosphine (1 M solution in THF, 2.21 mL). The reaction mixture was stirred at 50 °C for 2 hr. After cooling down to room temperature, the solution was evaporated under reduced pressure. The material was dissolved in a mixture of acetic acid (2 mL), water (6 mL) and methanol (6 mL). The solution was degassed by evacuating the air in the flask and refilling it with nitrogen five times. Palladium hydroxide on carbon (20%, Degussa type, 0.095 g) was added and the solution was charged with hydrogen. The reaction mixture was stirred at room temperature under hydrogen gas for 24 hr. The solution was filtered through Celite pad and concentrated. The residue was purified by chromatography on Amberlite CG-50 resin (NH_4^+ form) (2.5% concentrated ammonium hydroxide in water), concentrated, acidified with hydrochloric acid (pH ca. 4), and lyophilized to provide compound **39** (0.086 g, 73%) as a oily solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.50 (d, *J* = 4.0 Hz, 1H, H-1'). 3.79–3.73 (m, 3H, H-3', H-5, H-6'), 3.70–3.67 (m, 1H, H-6'), 3.59 (dd, *J* = 12.0, 6.5 Hz, 1H, H-5'), 3.52 (ca. t, *J* = 9.2, 1H, H-4), 3.46–3.39 (m, 2H, H-3, H-6), 3.30 (dd, *J* = 12.2, 6.6, 1H, H-2'), 3.22–2.15 (m, 1H, H-1), 2.35 (dt, *J* = 12.6, 4.3 Hz, 1H, H-2), 1.71 (q, *J* = 12.6 Hz, 1H, H-2) . ^{13}C NMR (150 MHz, $CDCl_3$) δ 97.1, 80.4, 74.7, 73.6, 72.3, 69.0, 60.3, 54.0, 49.6, 48.8, 28.3. HRMS (ESI, positive) calcd for $C_{12}H_{24}DN_3O_7^+$ [*M* + *H*]$^+$ 325.1829, found 325.1834.

S3. Supporting Figures

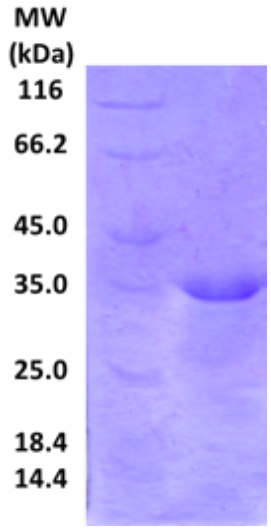


Figure S1. SDS PAGE of AprD3. The calculated molecular weight of AprD3 is 29.4 kDa

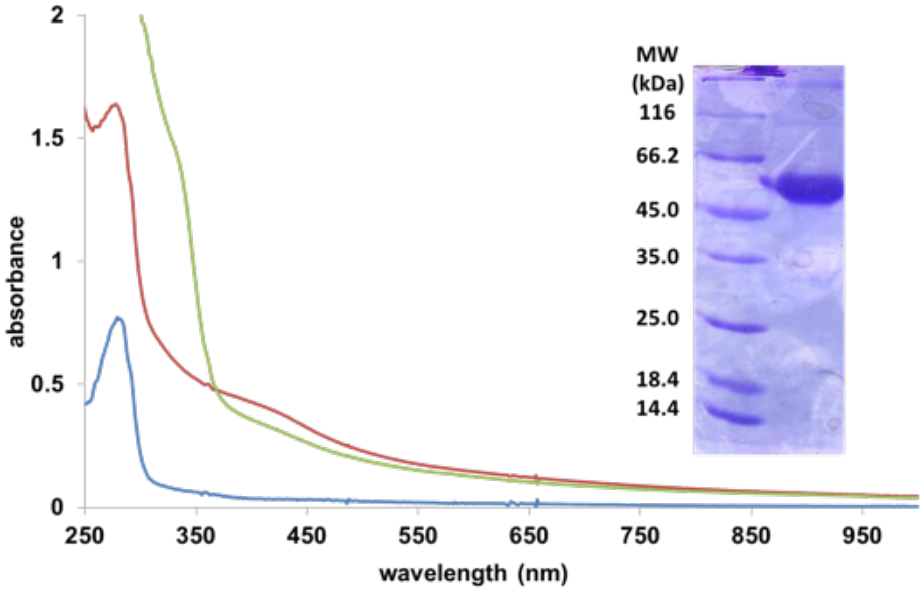


Figure S2. UV-visible absorption spectra and SDS PAGE of AprD4. The blue, red, and green spectra correspond to no reconstituted AprD4 (8 mM), reconstituted AprD4 (15 mM), and reconstituted AprD4 (15 mM) treated with 1 mM sodium dithionite. The bleaching of the absorbance shoulder at 420 nm is characteristic of a bound iron-sulfur cluster. The calculated molecular weight of AprD4 is 52.5 kDa.

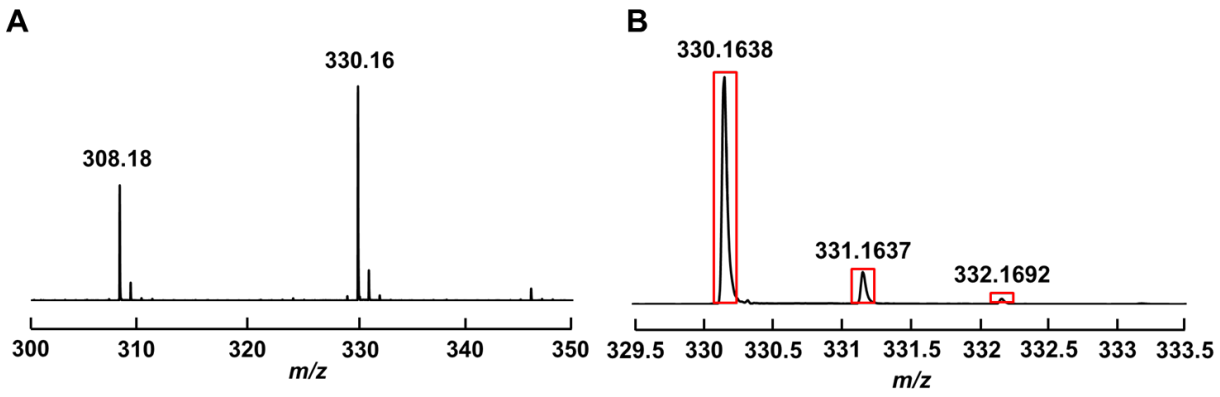


Figure S3. (A) Low resolution, and (B) high resolution mass spectra of the collected peak at 7.2 min shown in Figure 2B, trace g. (A) LRMS (ESI, positive) calculated for $C_{12}H_{25}N_3O_6^+$ $[M + H]^+$ 308.18 and $[M + Na]^+$ 330.16, observed 308.18 and 330.16, respectively. (B) HRMS (ESI, positive) calcd $[M + Na]^+$ 330.1636, observed 330.1638. These results were consistent with the chemical nature of lividamine (**13**).

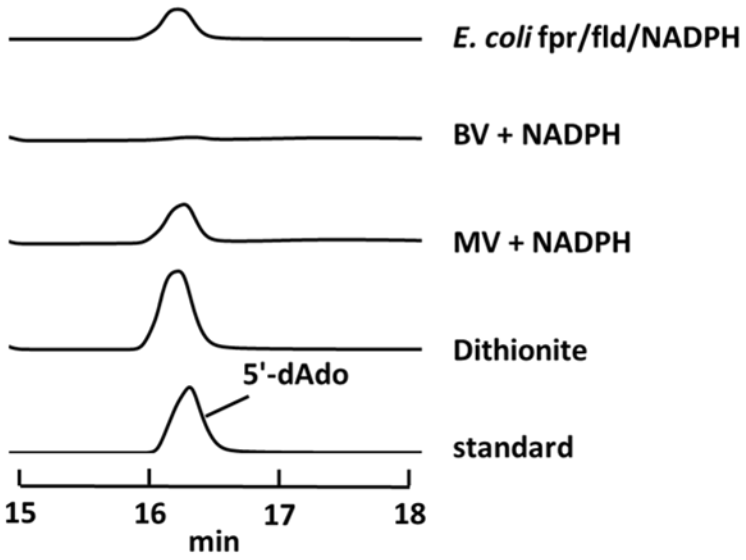


Figure S4. Competence of various reducing systems for activating AprD4. HPLC traces with UV detection at 260 nm showed 5'-dAdo (**19**) formation.

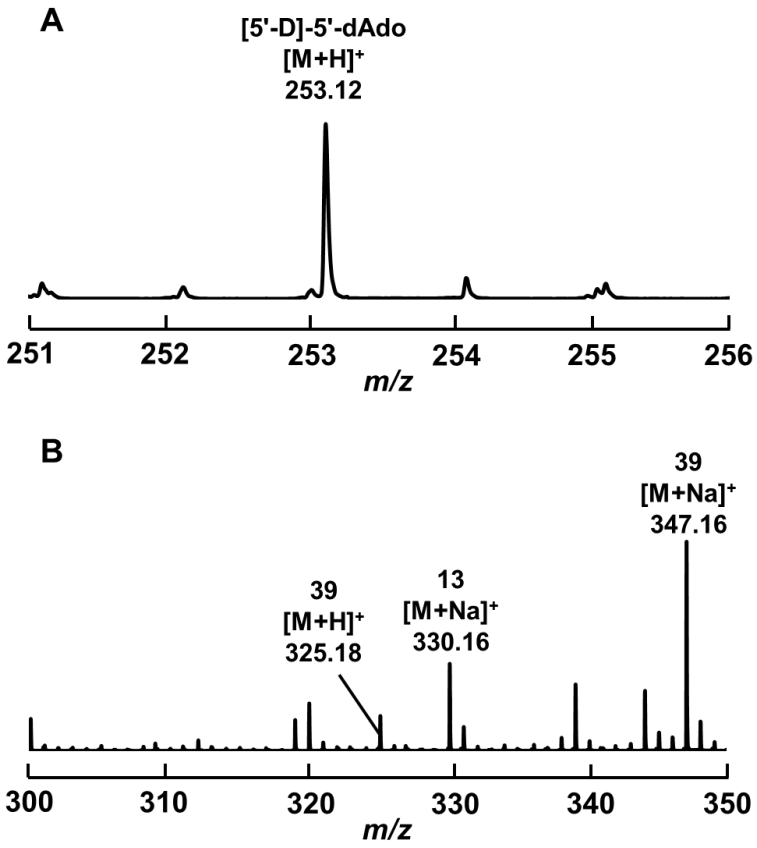


Figure S5. Mass spectra of (A) [5'-^{2}H]-5'-dAdo isolated from the incubation with [4'-^{2}H]-paromamine (**39**) with AprD4/AprD3 (B) assay mixture showing product (**13**) formation in the incubation of [4'-^{2}H]-paromamine (**39**) with AprD4/AprD3.

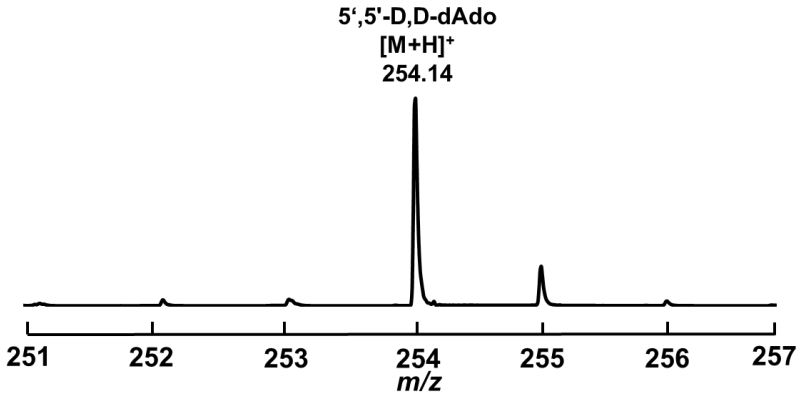


Figure S6. Mass spectra of [5'-$^2H_2$]-5'-dAdo produced by AprD4/AprD3 reaction with [5'-$^2H_2$]-SAM and unlabeled paromamine (**11**).

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